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Identification of Four Genes from the Granaticin Biosynthetic Gene Cluster of *Streptomyces violaceoruber* Tü22 Involved in the Biosynthesis of L-Rhodinose

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Four genes, ORF 22~25, from the granaticin biosynthetic gene cluster of *Streptomyces violaceoruber* Tü22 were analyzed for their involvement in the biosynthesis of the two deoxysugar moieties of the granaticins. Each gene was individually inactivated on a cosmid carrying the entire *gra* gene cluster and the mutant cosmids were transformed into *S. coelicolor* CH999. Analysis of the pattern of pigment production by the transformants revealed that each of the four ORFs is required for the formation/attachment of the L-rhodinose moiety of granaticin B, but not that of the D-olivose moiety of granaticin. Based on these results and sequence homologies a pathway of dTDP-L-rhodinose formation is proposed which implicates ORF23, and possibly also ORF 24, in the 3-deoxygenation reaction, ORF 25 in the epimerization and ORF 22 in the final 4-ketoreduction.

Dideoxyhexoses are frequently found as essential constituents of various classes of biologically active natural products, such as antibiotics, lipopolysaccharides or cardiac glycosides.[12,18,23,27,33,45,48,49] Despite their biological importance relatively little is known about their biosynthesis.[12,18,45] A notable exception is the both genetically and mechanistically well characterized formation of the 3,6-dideoxyhexose, ascarylose, in pathogenic bacteria such as *Salmonella* and *Yersinia* species.[6,11,12,19,25,35] We have been interested in the biosynthesis of the 2,6-dideoxyhexose moiety of the benzoisochromane quinone antibiotic, granaticin (**1**), and its direct precursor, dihydrogranaticin (**2**),[20] produced by *Streptomyces violaceoruber* Tü22, and that of the additional 2,3,6-trideoxyhexose, L-rhodinose, attached glycosidically to the first sugar in granaticin B (**3**) and its dihydro derivative **4**.[3] Feeding experiments with $^{14}C/^{3}H$-labeled glucose samples have established the origin of the carbon/hydrogen framework and have elucidated some stereochemical aspects of the formation of the sugar moiety of **1**.[39] Subsequently, the first two genes involved in the production of this deoxyhexose, encoding dTDP-glucose synthase and dTDP-glucose 4,6-dehydratase, were identified on a cosmid[4] which, as shown later, carried all the genes required for **1**, **2**, **3** and **4** biosynthesis.[17] However, no mechanistic information was available on the deoxygenation reactions in the biosynthesis of these or any other 2,6-dideoxyhexoses. While the sequencing of the entire *gra* cluster was in progress, we cloned four more deoxysugar biosynthesis genes from the cluster and analyzed their functions by gene inactivation experiments. All four of these genes turned out to be required for the 3-deoxygenation and other steps in the biosynthesis of the L-rhodinose moiety of **3** and **4**.

Results

Identification of Putative Deoxygenation Genes

Our original hypothesis assumed that the two deoxygenation reactions involved in the biosynthesis of the two sugar moieties of **1**~**4** may involve similar mechanisms and enzymes as the 3-deoxygenation in ascarylose biosynthesis.[27,42] Candidate genes from the granaticin cluster putatively involved in the deoxygenation reaction were located by Southern hybridization with two

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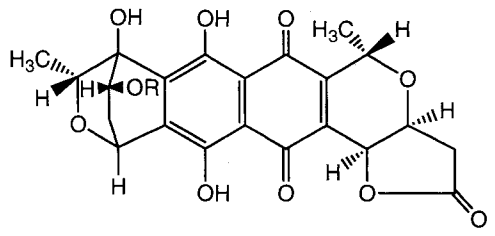
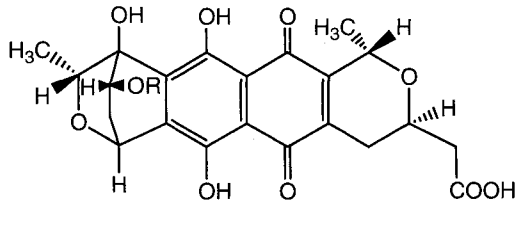


1: Granaticin (R = H)

3: Granaticin B (R = L-rhodinose)



2: Dihydrogranaticin (R = H)

4: Dihydrogranaticin B (R = L-rhodinose)

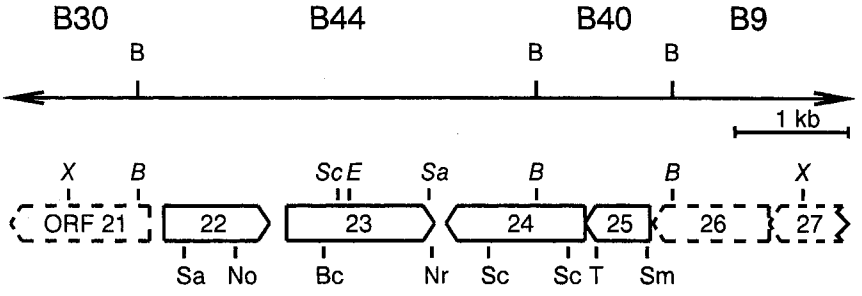
genes from a partial oleandomycin biosynthesis gene cluster cloned from *Streptomyces antibioticus* Tü99.[30,31] The deduced amino acid sequences of these two probe genes, *Tü99 orf5* and *orf9*, showed homology to the 3-deoxygenation enzyme E_1 (AscC) from *Y. pseudotuberculosis*[43] and to dTDP-4-keto-deoxysugar 3,5-epimerases, *e.g.*, StrM from *S. griseus*,[34] respectively. Positive hybridization signals with cosmid pOJ446-22-24[4] carrying the granaticin biosynthetic gene cluster[17] were localized to two *Bam*HI subclones, B40 (*Tü99 orf9*) and B44 (*Tü99 orf5*), respectively (Fig. 1). Sequencing of the 4.7 kb DNA fragment comprising B40 and B44 revealed four open reading frames, designated ORF 22~25 when this sequence became part of the complete sequence of the granaticin biosynthetic gene cluster.[17] ORF 23, which hybridized to *Tü99 orf5*, showed good homology to *ascC* and, unlike the probe gene, included coding sequences for a PMP (instead of PLP) binding site and an iron-sulfur cluster, consistent with its possible function in a deoxygenation reaction. The close homology of ORF 25 to other NDP-hexose 3,5-epimerases would be consistent with its function either as an epimerase in L-rhodinose formation or as a tautomerase converting dTDP-4-keto-6-deoxy-D-glucose to its 3-keto isomer, preparatory to 2-deoxygenation.

Gene Inactivation

In order to determine the functions of ORFs 23 and 25 in the biosynthesis of the granaticin sugar moieties, both open reading frames were inactivated individually. Likewise, the adjacent ORFs 22 and 24 were also disrupted to probe their possible involvement in deoxysugar biosynthesis. ORF 22 shows homology to various dTDP-hexose 4-ketoreductases, such as *dnmV* from *S. peucetius*,[29] including a highly conserved nucleotide binding motif.[17] ORF 24 shows no significant sequence homology to any genes in the databases and its function is unknown, but its translational coupling to the putative epimerase encoded by ORF 25 makes it a candidate gene for deoxysugar biosynthesis as well.

As reported previously,[17] granaticin and its derivatives are produced by heterologous expression of cosmids pOJ446-22-16 or pOJ446-22-24, harboring the granaticin biosynthetic gene cluster, in *S. coelicolor* A3 (2), strain CH999. Since the granaticin producer, *S. violaceoruber* Tü22, is difficult to manipulate genetically, and because it gives higher overall pigment yield and fewer side products we chose cosmid pOJ446-22-24 for gene disruption. Wild type genes were replaced by altered versions as described in the Experimental Section. Specifically, subclones harboring the genes of interest were used to engineer deletions of the central parts of the open reading frames depicted in Fig. 1. To avoid polar effects on downstream genes, especially in the case of the translationally coupled ORFs 24 and 25, in-frame deletions were constructed whenever possible. Each disrupted gene was introduced into the cosmid by homologous interplasmid recombination in *E. coli* according to a method published by BALASUBRAMANIAN *et al.*[2] The presence of the expected deletion was confirmed by restriction analysis of each mutated cosmid with *Kpn*I or *Bam*HI (Fig. 2). The deletion in ORF 22 (cosmid pDT36) removed two *Kpn*I sites, resulting in a fusion to a 10385 nt fragment. The 3511 nt *Bam*HI fragment of the wild type shifted to 3055 nt as expected for replacement of ORF 22 with its altered derivative. The deletion in ORF 23 (pDT28) resulted in a 5879 nt *Kpn*I fragment and a 2559 nt *Bam*HI fragment. Disruption of ORF 24 (pDT23) caused a shift of the *Kpn*I band to 6060 nt and removal of a *Bam*HI site, resulting in a merged *Bam*HI fragment of 3926 nt. In the

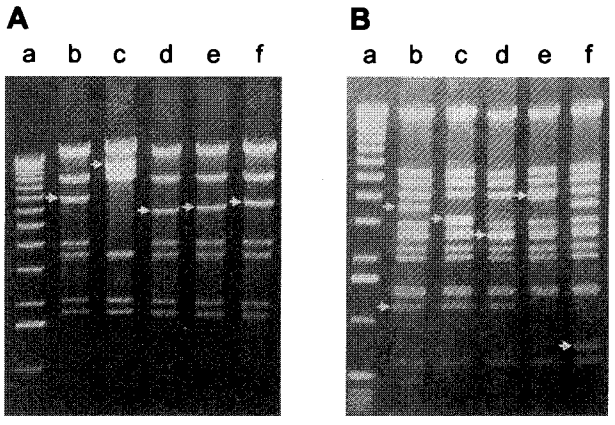
Fig. 1. Organization of the part of the granaticin biosynthetic gene cluster of *S. violaceoruber* Tü22 used for the construction of deletions of the genes described in this work (indicated by open arrows: 22 4-ketoreductase, 23 3-dehydrase, 24 unknown, 25 3,5-epimerase).



Dashed arrows refer to adjacent ORFs. The location of the restriction sites used to generate the mutations are shown below each gene. Restriction sites limiting the deletion constructs are indicated above the genes.

Abbreviations: B, *Bam*HI; Bc, *Bcl*I; E, *Eco*RI; No, *Not*I; Nr, *Nru*I; Sa, *Sal*I; Sc, *Sac*I; Sm, *Sma*I; T, *Tfi*I; X, *Xma*I.

Fig. 2. Ethidium bromide-stained agarose gels of DNA from cosmid pOJ446-22-24 and derivatives harboring deletions in genes ORF 22, 23, 24 or 25.



(A) Restriction with *Kpn*I. (B) Restriction with *Bam*HI. Lane a, 1 kb ladder; lane b, pOJ446-22-24; lane c, cosmid pDT36 (deletion in ORF 22); lane d, cosmid pDT28 (deletion in ORF 23); lane e, cosmid pDT23 (deletion in ORF 24); lane f, cosmid pDT26 (deletion in ORF 25). Fragments carrying genes of interest are indicated with arrows.

ORF 25 deletion (pDT26) 6384 nt and 739 nt fragments were detected after *Kpn*I and *Bam*HI digestion, respectively.

Analysis of the Mutated Cosmids

Heterologous expression of the granaticin cluster harboured by cosmid pOJ446-22-24 in *S. coelicolor* CH999 yielded almost equal amounts of dihydrogranaticin and dihydrogranaticin B as well as traces of granaticin and granaticin B, representing the four end products of the granaticin pathway.[17] To establish the effects of the deletions on pigment production, the mutated cosmids were transformed into *S. coelicolor* CH999 and the cells were grown as described in the Experimental Section. After extraction of pigments, analytical TLC (data not shown) and analytical HPLC were performed using purified compounds as standards. As shown in Figure 3, granaticin was detected as the main product from every single mutant. Except for pDT36, smaller amounts of the direct precursor dihydrogranaticin were also detected. The synthesis of dihydrogranaticin B and the corresponding lactone granaticin B was completely abolished by the deletions in ORF 23 or ORF 24, whereas only traces of these compounds were produced after disrupting ORF 22 or ORF 25. These results were confirmed by mass spectrometry. Specifically, apart from granaticin (M-1: 443) and dihydrogranaticin (M-1: 445) anion electrospray mass spectrometry revealed traces of granaticin B (M-1: 557) and dihydrogranaticin B (M-1: 559) in the case of disruption of ORF 25 while only granaticin B was detected in traces when ORF 22 was inactivated. The latter result was further confirmed by LC-electrospray mass spectrometry.

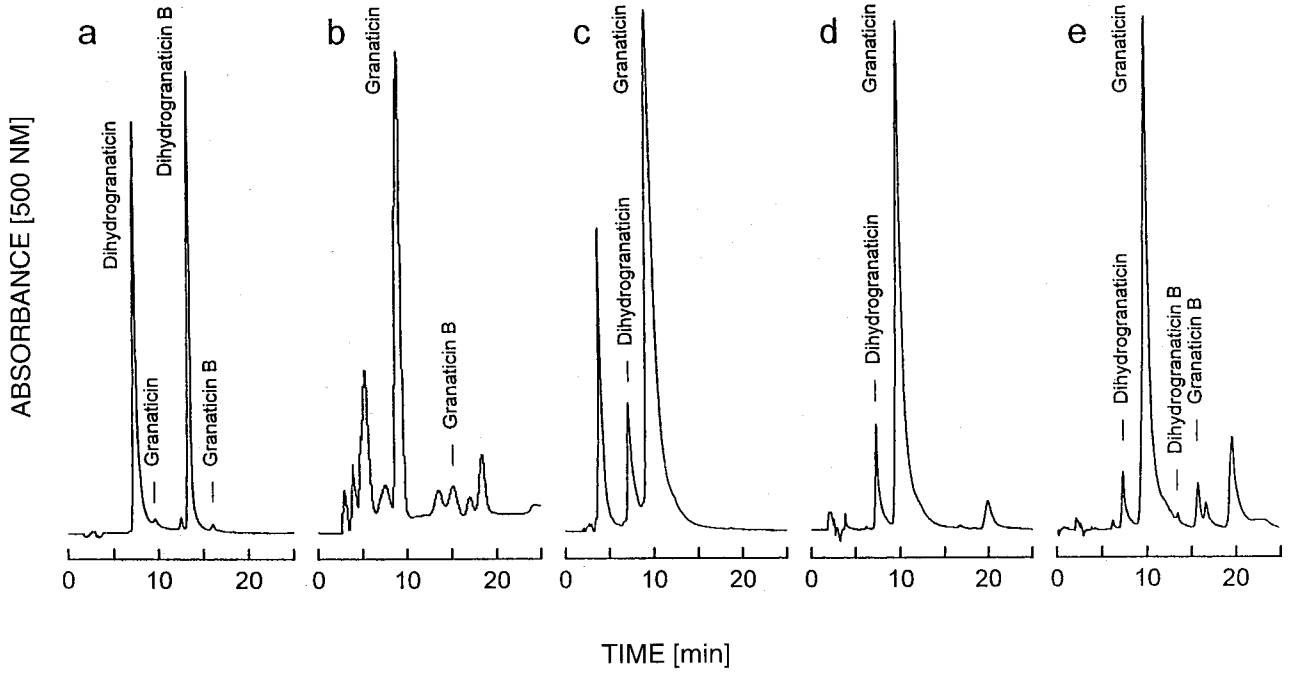
Table 1. Selected plasmids and cosmids used in this study.

Plasmid/Cosmid	Description	References
Plasmid		
B30, B44, B40, B9	BamHI-subclones of pSK(-) harboring inserts (2.5 kb, 3.5 kb, 1.2 kb and 2.9 kb, respectively) of a consecutive stretch of *S. violaceoruber* Tü22 genomic DNA which contains genes for granaticin biosynthesis.	4
pDT19	Derivative of pCVD446 containing an in-frame deletion of 771 nt in ORF 24.	This study
pDT22	Derivative of pCVD446 containing an in-frame deletion of 447 nt in ORF 25.	This study
pDT27	Derivative of pCVD446 containing an out-of-frame deletion of 952 nt in ORF 23.	This study
pDT35	Derivative of pCVD446 containing an in-frame deletion of 456 nt in ORF 22.	This study
Cosmid		
pOJ446-22-24	A derivative of cosmid pOJ446 (ref. 5) containing the whole set of genes from *S. violaceoruber* Tü22 necessary to produce granaticin metabolites in a heterologous host.	4, 17
pDT23	A derivative of cosmid pOJ446-22-24 containing a deletion in ORF 24.	This study
pDT26	A derivative of cosmid pOJ446-22-24 containing a deletion in ORF 25.	This study
pDT28	A derivative of cosmid pOJ446-22-24 containing a deletion in ORF 23.	This study
pDT36	A derivative of cosmid pOJ446-22-24 containing a deletion in ORF 22.	This study

Discussion

In recent years increasing knowledge of the biosynthesis of di- and trideoxysugars as essential constituents of biologically active natural products has been gained. This was achieved mainly by DNA sequence analysis, comparison of deduced amino acid sequences with proteins of known function and determination of phenotypes of mutants after targeted gene disruption. In order to identify the functions of four genes from the granaticin biosynthetic gene cluster we carried out targeted inactivations of ORFs 22, 23, 24 and 25. The results, complete or almost complete abolishment of (dihydro)-granaticin B formation, but continued production of (dihydro)granaticin, provide unambiguous evidence that the gene products of ORFs 22~25 from the granaticin

Fig. 3. HPLC analysis of granaticins produced by *S. coelicolor* A3(2) strain CH999 transformed with (a) pOJ446-22-24, (b) pDT36 (deletion in ORF 22), (c) pDT28 (deletion in ORF 23), (d) pDT23 (deletion in ORF 24) and (e) pDT26 (deletion in ORF 25).

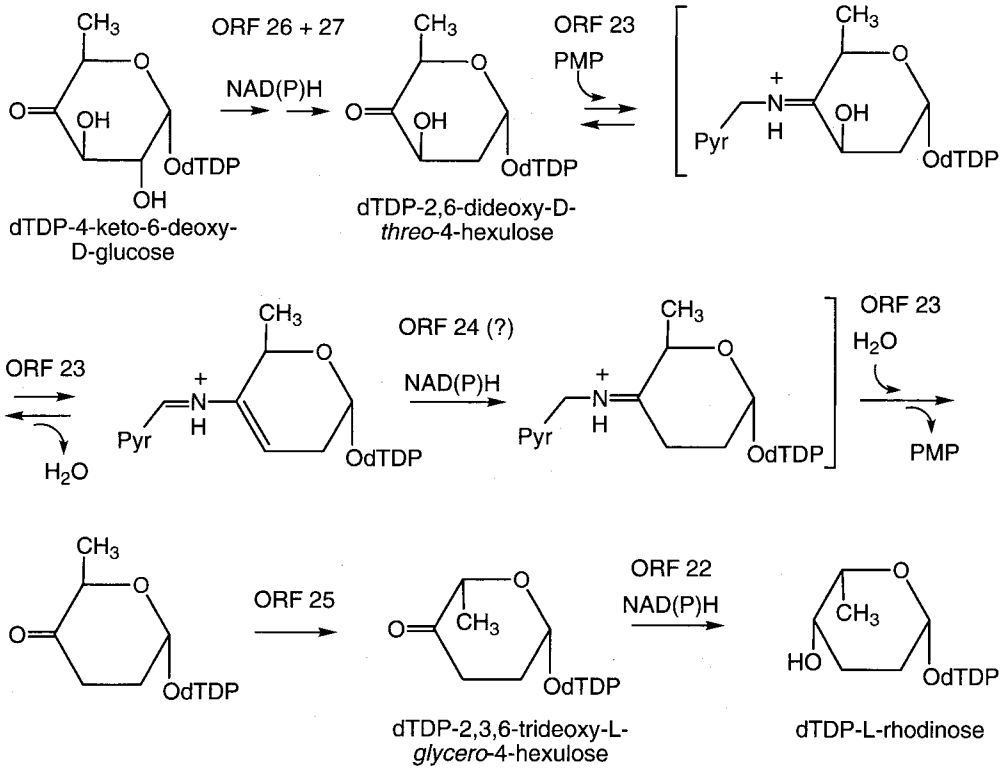


HPLC conditions and retention times are given in the Experimental Section.

biosynthetic gene cluster are specifically required for the biosynthesis or attachment of the L-rhodinose moiety of (dihydro)granaticin B, but not of the 4-keto-D-olivose moiety of (dihydro)granaticin. The presence of traces of **3** and/or **4** in the mutants lacking functional ORF 22 (4-ketoreductase) and ORF 25 (3,5-epimerase) is probably due to low level complementation by host genes and spontaneous epimerization, respectively. However, the drastic reduction in production of **3** and **4** leaves no doubt that these two genes, too, are essential for L-rhodinose formation or attachment. The mutations in all the plasmids also resulted in a dramatic shift from the predominant production of dihydrogranaticin **2** and dihydrogranaticin B **4** in the transformants expressing the wild-type plasmid pOJ446-22-24 to formation of a much higher proportion of granaticin **1** than its dihydro derivative **2**. Such changes in this ratio, which may reflect changes in the redox state of the system, were also observed when expression of pOJ446-22-24 was compared with that of the slightly shorter plasmid pOJ446-22-26 and the original producer, *S. violaceoruber* Tü22.[17] The underlying cause is not understood at this time.

The determination that ORFs 22~25 are not involved in 4-keto-D-olivose formation led directly to the recognition of ORF 26 and ORF 27[17] as the only other candidate genes in the cluster for the 2-deoxygenation step, setting the stage for the discovery of the true 2-deoxygenation mechanism.[9] This involves the 2,3-dehydration of dTDP-4-keto-6-deoxy-D-glucose by the ORF 27 gene product to give an unstable 2-deoxy-3,4-diketo intermediate, which is then trapped by reduction to dTDP-2,6-dideoxy-D-*threo*-4-hexulose by the 3-ketoreductase encoded by ORF 26. ORF 27 is homologous to proposed[29,36,41] 2,3-dehydratase genes in biosynthetic gene clusters for several other antibiotics containing 2-deoxysugars, such as *dnmT* from the daunorubicin cluster of *S. peucetius*,[38] *eryBVI* from the erythromycin cluster of *Sac. erythrea*,[10,41] *tylX3* from the tylosin cluster of *S. fradiae*,[7] *lanS* from the landomycin cluster of *S. cyanogenus* S136[47] or *oleV/orf10* from the oleandomycin cluster of *S. antibioticus*.[1,9,30] The 2-deoxygenation mechanism demonstrated with Gra ORF 26+27 has been confirmed at the biochemical level with the gene products of *tylX3*[7] and *Tü99 orf10*[9] and their 3-ketoreductase partners, *tylC1* and *Tü99 orf11*, respectively.

Fig. 4. Proposed pathway for the formation of dTDP-L-rhodinose from dTDP-4-keto-6-deoxy-D-glucose in the biosynthesis of granaticin B.



The identification of the role of ORFs 22~25 in the biosynthesis of the granaticins enables us to propose a detailed pathway for the formation of dTDP-L-rhodinose, the precursor of the 2,3,6-trideoxyhexose moiety of granaticin B, as shown in Fig. 4. Since the cluster contains only one set of the genes required to synthesize dTDP-4-keto-6-deoxy-D-glucose (ORF 16+17) and to carry out the 2-deoxygenation (ORF 26+27), it follows that these steps must be shared by the pathways to both sugar moieties, *i.e.*, dTDP-2,6-dideoxy-D-*threo*-4-hexulose must not only be the substrate for the transfer of the sugar moiety to the aglycone of granaticin but also the starting material for the formation of dTDP-L-rhodinose. The functions of the four genes ORF 22~25 can then be reasonably assigned to the necessary reactions based on their sequence homologies and the available biochemical data.

The gene product of ORF 23 shows significant similarity to deduced amino acid sequences of a family of genes which mostly seem to be involved in dehydration and transamination reactions, primarily in the formation of deoxysugar moieties of secondary metabolites.[32,33,42] More specifically, it belongs to a subgroup of genes of this family[22,32] which encode enzymes containing PMP and an iron-sulfur cluster and are known or proposed to be involved in 3-deoxygenation reactions of deoxysugars, including the well characterized enzyme E1 (AscC) from *Y. pseudotuberculosis*. The fact that the enzyme encoded by ORF 23 is required for (dihydro)granaticin B, but not granaticin formation provides strong evidence for its function as a dTDP-2,6-dideoxy-4-ketoglucose 3-dehydrase in L-rhodinose biosynthesis. Hence the 3-deoxygenation reaction in L-rhodinose biosynthesis in *S. violaceoruber* Tü22 seems to occur by a similar mechanism as the 3-deoxygenation in ascarylose biosynthesis catalyzed by the combined action of the enzymes E1 (AscC) and E3 (AscD).[12,18,27] E3, which also contains an iron-sulfur cluster, serves as an electron transfer protein delivering reducing equivalents from NAD(P)H to E1, a process which seems to involve direct protein-protein interaction.[8] However, since the *gra* cluster does not contain a homolog of *ascD*, a different enzyme and possibly a different mechanism may be responsible for delivering reducing

equivalents to the 3-dehydrase encoded by ORF23. Alternatively, a general electron-transfer protein encoded outside the *gra* cluster may serve as the reductase, a possibility supported by the observation[46] that extraneous electron transfer proteins, such as diaphorase or the reductase component of methane monooxygenase, can work with E1 in place of E3.

The start codon ATG of ORF 24 overlaps with the stop codon TGA of the adjacent ORF 25, pointing to translational coupling of these two genes. This suggests that ORF 24 may have a function related to that of ORF 25, a fact confirmed by the conclusion from the gene inactivation experiments that both genes are required for L-rhodinose biosynthesis. However, amino acid sequence comparison revealed no meaningful similarity to current database entries and thus provided no further clues to this function. It is possible that ORF 24 is required for the transfer of the L-rhodinose moiety from dTDP-L-rhodinose to the 3′ oxygen of granaticin to form granaticin B. However, a good candidate gene to encode this *O*-glycosyltransferase, ORF 14, has already been identified in the *gra* cluster.[4,17] Alternatively, ORF 24 could encode a reductase needed for completion of the 3-deoxygenation reaction catalyzed by ORF 23, in analogy to the function of E3. As yet another possibility, ORF 24 may be a regulatory gene specifically controlling the (dihydro)granaticin B branch of the overall biosynthetic pathway. Clearly, its specific role in L-rhodinose biosynthesis needs further investigation.

As reported previously,[17] the deduced amino acid sequence of ORF 25 shows similarity with those of genes from sugar biosynthetic pathways postulated to encode 3(5)-epimerases. This includes putative dTDP-4-keto-6-deoxyglucose 3,5-epimerases from *S. griseus* (*strM*),[34] *S. nogalater* (*snoF*),[44] *S. antibioticus* (oleL/Tü99 *orf9*)[1,30,31] and *E. coli* (*rfbC*),[40] as well as 5-epimerases from *Y. pseudotuberculosis* (*ascE*)[43] and *Sac. erythraea* (*eryBVII*).[41] These 3(5)-epimerases are suggested to invert the configuration of C-3 hydroxyl groups and/or C-5 methyl groups of NDP-4-ketohexoses which are common intermediates in the biosynthesis of many deoxysugars. The targeted mutation of ORF 25 clearly proved its involvement in L-rhodinose biosynthesis. All the data are consistent with the conclusion that dTDP-2,6-dideoxy-D-*threo*-4-hexulose must be the common precursor for both deoxysugar moieties, and 2-deoxygenation must therefore precede epimerization. However, the available information does not reveal the order in which the 3-deoxygenation and the epimerization steps occur, *i.e.*, whether dTDP-2,6-dideoxy-D-*threo*-4-hexulose or dTDP-2,3,6-D-*glycero*-4-hexulose is the natural substrate of the ORF 25 gene product. The pathway depicted in Fig. 4 shows 3-deoxygenation prior to 5-epimerization, in analogy to ascarylose formation,[27] but the order of these two steps could also be reversed. In either case, the enzyme acting immediately after the epimerase, *i.e.*, either the 4-ketoreductase or the 3-dehydrase, must drive the reaction by virtue of its substrate specificity, selecting from the mixture of epimers only the one which leads to the intended product, L-rhodinose.

The final step of the biosynthesis of L-rhodinose is likely to be mediated by the gene product of ORF 22. Its deduced amino acid sequence displays similarity to several UDP-glucose 4-epimerases such as *galE* encoding the UDP-galactose 4-epimerase from *E. coli*[26] and to genes encoding putative NDP-4-keto-deoxyhexose ketoreductases which are involved in 2,3,6-trideoxysugar biosynthesis. These include *dnmV* from *S. peucetius*[29] and ORF 5 from *S. griseus*[24] for daunosamine biosynthesis and *snoG* from *S. nogalater* for the biosynthesis of rhodosamine and 2-deoxy-L-fucose.[44] Less homology is shared with CDP-abequose synthase[21] and CDP-paratose synthase[14] from *Y. pseudotuberculosis*, both involved in 3,6-dideoxyhexose biosynthesis. The common feature of all these proteins is that they contain a rigorously conserved dinucleotide-binding domain and catalyze oxidations or reductions at C-4 of nucleotide-activated hexoses. These data together with its identification as a L-rhodinose biosynthetic gene by the gene inactivation experiment strongly suggest that ORF 22 encodes a dTDP-2,3,6-trideoxy-L-*glycero*-4-hexulose 4-ketoreductase.

Although this remains to be proven, it seems likely that the four genes described here represent all the genetic information required for the conversion of dTDP-2,6-dideoxy-D-*threo*-4-hexulose to dTDP-L-rhodinose. A similar set of genes for which the same functions are proposed has been found in the urdamycin biosynthetic gene cluster of *S. fradiae*.[15] The recent establishment of an enzymatic system for the synthesis of dTDP-2,6-dideoxy-D-*threo*-4-hexulose from dTDP-4-keto-6-deoxy-D-glucose[9] using the ORF 26 and 27 gene products has paved the way for an evaluation of this assumption at the biochemical level, and work along these lines is in progress.

Experimental

Bacterial Strains and Plasmids

Escherichia coli XL1-Blue (Stratagene) and DH5α[13] were used for routine subcloning of restriction fragments. JM110[50] served to obtain demethylated plasmid-DNA for digestion of restriction sites which are blocked by

overlapping *dam* methylation (BclI, NruI for deletion of ORF 23). Strains RR1, SY327-λ (*pir*) and SM10-λ (*pir*) as well as suicide vector pCVD442 for interplasmid recombination were a gift from W. R. JACOBS, Jr.[2] *E. coli* ET12567/pUZ802 and *S. coelicolor* A3(2) CH999 were obtained from D. A. HOPWOOD.[28] pOJ446-22-24[4] is a derivative of cosmid pOJ446[5] containing the whole set of genes from *S. violaceoruber* Tü22 necessary to produce granaticin metabolites in a heterologous host.[17] Plasmids pBluescriptSK (−) (pSK (−)) and pUC118 were purchased from Stratagene. Other plasmids and cosmids used in this study are listed in Table 1.

Culture Conditions and DNA Manipulations

For plasmid propagation *E. coli* strains were routinely grown at 37°C in Luria-Bertani (LB) broth or on LB agar supplemented with 100 μg/ml carbenicillin. Plasmid preparation and culture conditions for *Streptomyces* were described previously.[16] General *in vitro* DNA manipulations were performed according to standard protocols.[37] DNA fragments for subcloning were isolated from agarose gels with the Qiaquick gel extraction kit (Qiagen).

Gene Disruption and Analysis of Mutants

To determine the role of ORFs 22~25 in the biosynthesis of granaticin gene disruption experiments were carried out by replacing wild type genes located on cosmid pOJ446-22-24 with altered derivatives. For homologous recombination each deletion is flanked by 0.9~1.7 kb of contiguous DNA. Fragments containing the altered genes were cloned into suicide vector pCVD442 and maintained in *E. coli* SY327-λ (*pir*). The generation of the individual deletion constructs is described below (*cf.* Fig. 1).

Inactivation of ORF 22

The 0.4 kb *Bam*HI-*Sal*I fragment with a blunt-ended *Sal*I site and the 1.7 kb *Not*I-*Sal*I fragment with a blunt-ended *Not*I site, both originating from B44, were ligated into the *Bam*HI/*Sal*I sites of pSK(−). This caused a 456 nt in-frame deletion which removes codons 53~204 from ORF 22. For extension of the flanking DNA this newly created *Bam*HI-*Sal*I fragment was combined with the adjacent 0.6 kb *Xma*I-*Bam*HI fragment from B30 by ligation into pSK(−) restricted with *Sal*I/*Xma*I. Digestion with *Kpn*I/*Sac*I, both located in the multicloning site of pSK(−), followed by treatment with the Klenow fragment of DNA polymerase I and ligation of the insert into the *Sma*I site of pCVD442 resulted in plasmid pDT35.

Inactivation of ORF 23

Consecutive restriction of demethylated B44 with *Bcl*I and *Nru*I, where the *Bcl*I site was blunt-ended by Klenow treatment, followed by religation produced an out-of-frame deletion of 952 nt which starts at codon 111. Digestion with *EcoRI*/*Xba*I, both located in the multicloning site flanking the insert, followed by treatment with the Klenow fragment of DNA polymerase I and ligation of the insert into the *Sma*I site of pCVD442 resulted in plasmid pDT27.

Inactivation of ORF 24

Assembling of the 0.9 kb *Sac*II-*Bam*HI fragment of B40 and the 1.3 kb *Sac*II fragment from B44 created an in-frame deletion of 771 nt which removes codons 49~305. The correct orientation of the *Sac*II fragment was checked by restriction analysis with *Apa*I. The insert of the resulting plasmid was obtained by digestion with *Eco*NI and *Bam*HI. Subsequent Klenow treatment and ligation into the *Sma*I site of pCVD442 created plasmid pDT19.

Inactivation of ORF 25

The 0.5 kb *Bam*HI-*Tfi*I fragment with a blunt-ended *Tfi*I site and the 0.2 kb *Sma*I-*Bam*HI fragment both originating from B40 were ligated into the *Bam*HI site of pSK(−). This created an in-frame deletion of 447 nt which removes codons 11~159 of ORF 25. To extend the flanking region on both sides of the deletion the 1.0 kb *Sal*I-*Bam*HI fragment from B44 and the 1.1 kb *Bam*HI-*Xma*I fragment from B9 were ligated together into pUC118 restricted with *Sal*I and *Xma*I. Subsequently, the 0.7 kb *Bam*HI fragment carrying the deletion was ligated into the *Bam*HI site of this plasmid. The complete insert was obtained by digestion with *Eco*RI and *Pst*I, both located in the multicloning site of pSK(−), treated with the Klenow fragment of DNA polymerase I and ligated into the *Sma*I site of pCVD442 resulting in plasmid pDT22.

Conjugation and Selection for Plasmid-cosmid Recombination

The method followed for replacement of wild type genes located on cosmid pOJ446-22-24 with disrupted derivatives was that published by BALASUBRAMANIAN *et al.*[2] Because no resistance cassette was inserted into the altered gene which would allow direct screening for double crossover events the protocol was modified regarding selection for single crossovers. Specifically, single colonies of donor cells SM10-λ (*pir*) transformed with deletion constructs pDT35, pDT27, pDT19 or pDT22 and acceptor strain RR1 containing cosmid pOJ446-22-24 were grown for 12~18 hours in LB medium supplemented with carbenicillin or

apramycin, respectively (100 μg/ml each). Aliquots of each culture (500 μl each) were mixed with 5 ml of 10 mM $MgSO_4$ by vortexing and centrifuged. The pellets were resuspended in 100 μl $MgSO_4$, spotted onto LB plates supplemented with 100 μg/ml carbenicillin, 100 μg/ml apramycin and 500 μg/ml streptomycin (for selection against streptomycin-sensitive donor cells) and incubated at 37°C. After 18 to 24 hours the cells were pooled in 2 ml of 10 mM $MgSO_4$ and 100 μl were inoculated into 10 ml of LB medium containing carbenicillin, apramycin and streptomycin. After incubation at 37°C for 4 hours the cells were pelleted, spread onto LB plates with all three antibiotics and grown for 18 hours. Then, all colonies from one plate were pooled into 1~2 ml of LB medium and plasmid DNA was prepared. *E. coli* DH5α was transformed with these plasmid preparations and plated onto LB plates containing carbenicillin and apramycin. Cells growing under these conditions should contain co-integrates with one wild type and one disrupted gene copy due to single crossover events. Single colonies from these plates were inoculated into 2 ml LB medium with 100 μg/ml apramycin and cultivated overnight to allow double crossover. Aliquots from the overnight cultures (10 and 100 μl) were plated onto LB agar supplemented with apramycin and 5% (wt/vol) sucrose to select against single crossover recombinants containing the *sacB* gene from vector pCVD442, whose expression makes the cells sensitive to sucrose. After incubation for 12~18 hours single colonies from these plates were screened for double homologous recombination resulting in the replacement of the targeted genes by confirming the predicted restriction pattern of *Bam*HI and *Kpn*I fragments, respectively. Derivatives of cosmid pOJ446-22-24 were introduced into *S. coelicolor* CH999 by conjugation from *E. coli* ET12567/pUZ802 as described previously,[5,17] except that R2YE plates were used for growth on solid media. When selection was required, 100 μg/ml apramycin sulfate and 25 μg/ml nalidixic acid were added.

Analysis of Mutants

Pigment production of transformants of *S. coelicolor* CH999 with derivatives of pOJ446-22-24 carrying deletions in ORF 22 (cosmid pDT36), ORF 23 (pDT28), ORF 24 (pDT23) or ORF 25 (pDT26) was analyzed by analytical TLC on 0.2 mm silica gel plates (Kieselgel 60 F_{254}, Merck) according to SNIPES *et al.*[39] Further, analytical HPLC with an Econosil C-18-5U column (5 m, 250×4.6 mm, Alltech) and anion electrospray mass spectrometry for determination of molecular mass (Fisons VG Quattro II mass spectrometer) were carried out as described by ICHINOSE *et al.*[17] Rf values and retention times corresponded to those published previously,[17] except for crude extracts of transformants of CH999 with pDT36 because these experiments were done with a slightly different column (TSK ODS-120T, 5 m, 250×4.6 mm, TosoHaas; retention times: dihydrogranaticin, 8.17 minutes; granaticin, 9.30 minutes; dihydrogranaticin B, 14.19 minutes, granaticin B, 15.37 minutes). Additionally, crude extracts of CH999 with pDT36 were analyzed by HPLC-electrospray mass sprectrometry (Fisons VG Platform 1 mass spectrometer). The HPLC chromatogram was developed with the same column (0~8 minutes H_2O/acetonitrile 60 : 40, 8~45 minutes 55 : 45, flow rate 0.5 ml/minute, absorbence 500 nm) at room temperature. The retention times of the compounds of interest were as follows: dihydrogranaticin, 6.8 minutes; dihydrogranaticin B, 8.6 minutes; granaticin, 13.1 minutes; granaticin B, 17.9 minutes. Purified compounds were used as standards for coinjection with crude extracts.

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